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Detection of the principal synthetic route indicative impurity in Lamotrigine

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Abstract

An analytical method has been developed for the detection of trace amounts of the principal synthetic route indicative impurity in lamotrigine (3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine). A sample extract was preconcentrated by normal-phase high-performance liquid chromatography (HPLC) and analysed by subsequent on-line reversed-phase HPLC–thermospray mass spectrometry (TSP-MS). During the sample extraction and concentration step, carried out by semipreparative normal-phase chromatography, the preliminary separation of the impurity from the lamotrigine takes place. The organic solvent (dichloroethane–methanol, 90:10, v/v) is evaporated from the collected fraction and the material is redissolved in a smaller volume of the reversed-phase mobile phase. The collected fraction is then subjected to reversed-phase HPLC–TSP-MS. The influence of an ultrasonic extraction step has been examined. When the method was applied to lamotrigine tablets, a shake flask partitioning step using 1 mg/ml EDTA in water–dichloroethane was used instead of the ultrasonic extraction. Detection limit and recovery measurements showed that the route indicative impurity formed during the synthesis could be detected in the 50–100 ppb (w/w) range.

Keywords: Lamotrigine; Route indicative impurity; HPLC; TSP-MS

1. Introduction

3,5-Diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine (lamotrigine) is the active ingredient in the antiepileptic product manufactured and marketed by GlaxoWellcome under the trademark Lamictal (see compound **1** in Fig. 1). Lamotrigine is a novel antiepileptic that has a membrane-stabilising mechanism via blockade of voltage-dependent sodium channels and inhibition of glutamate release (Leach et al., 1986; Miller et al., 1986). Its efficacy as add-on therapy has been demonstrated

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in both children (Battino et al., 1993; Schlumberger et al., 1994) and adults (Jawad et al., 1989; Loiseau et al., 1990).

Several methods for the study of lamotrigine and its metabolites in biological matrices have been reported; these have included use of liquid chromatography–thermospray mass spectrometry (Doig and Clare, 1991), reversed-phase HPLC after extraction (Yamashita et al., 1995; Fraser et al., 1997; Hart et al., 1997), capillary electrophoresis (Shihabi and Oles, 1996), normal-phase HPLC (Fazio et al., 1992; Sallustio and Morris, 1997), gas chromatography with nitrogen phosphorus detection (Watelle et al., 1997) and gas chromatography–mass spectrometry after conversion to a *tert*.-butyldimethylsilyl derivative (Dasgupta and Hart, 1997).

As our purpose was to develop a method for the detection of the principal route indicative impurity, (*Z*)-2-(2,3-dichlorophenyl)-2-(guanidinylimino)acetonitrile (14W80, **2** in Fig. 1), present at low concentration in lamotrigine, a single reversed-phase procedure without preconcentration could not be applied. The compound of interest (14W80) is more hydrophobic than lamotrigine, and therefore elutes with a longer retention time on a reversed-phase column. This is disadvantageous for low level detection. A volatile buffer such as ammonium acetate should be present in the mobile phase for thermospray (TSP) buffer ionisation. The use of TSP ionisation with ammonium acetate buffer had been reported (Doig and Clare, 1991) using a gradient HPLC system for identification of metabolites, and so thermospray was chosen as a suitable ionisation mode. The involatile phosphate buffer used in other HPLC methods (Yamashita et al., 1995; Hart et al., 1997) is not applicable to this technique. The presence of magnesium stearate and iron oxide in the tablet formulation strongly affects the extraction efficiency of 14W80, and so a shake flask partitioning method, using EDTA to preferentially complex the metals had to be developed.

In this paper an analytical method is described by which approximately 50–100 ppb (w/w) of the route indicative impurity, 14W80, can be detected for purposes of patent protection.

2. Materials and methods

The compound of interest, 14W80 (**2** in Fig. 1) was synthesised by GlaxoWellcome (Dartford, UK). The purity of 14W80 was checked by RP-HPLC–thermospray mass Spectrometry. The methanol and 1,2-dichloroethane used were HPLC grade obtained from Rathburn (Walkerburn, UK). The ammonium acetate and ethylenediaminetetraacetic acid (EDTA) were HPLC grade and obtained from Fisons (Loughborough, UK).

2.1. Sample preparation and preconcentration from lamotrigine bulk powder

A total of 200 mg of bulk lamotrigine powder was weighed into a scintillation vial, 20 ml of extraction solvent were then added to the vial ('the sample extraction vial'). Another 20 ml of extraction solvent was added to a separate vial and subjected to the same procedures to provide an extraction blank. The extraction solvent was 1,2-dichloroethane–methanol (90:10, v/v). This

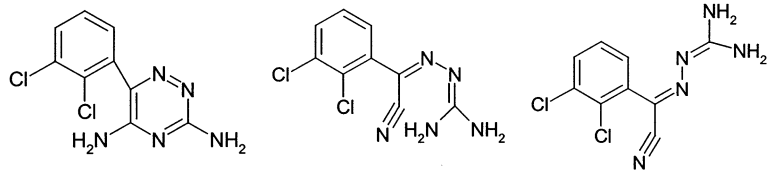


Fig. 1. Chemical structures of the compounds investigated. (**1**) 3,5-Diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine (Lamotrigine); (**2**) (*Z*)-2-(2,3-dichlorophenyl)-2-(guanidinylimino)acetonitrile (14W80); (**3**) (*E*)-2-(2,3-dichlorophenyl)-2-(guanidinylimino)acetonitrile (13W80).

solvent mixture was used as the mobile phase in the normal-phase chromatography. Both vials were placed in an ultrasonic bath for 15 min and then on a mechanical shaker for 30 min. After centrifuging both vials for 30 min at 2500 rpm, the supernatant liquid from the sample extraction vial was removed and transferred to another vial. The contents of both vials were concentrated under a stream of dry nitrogen until approximately 1 ml remained. The two vials were centrifuged again at 2500 rpm for 15 min. The supernatant liquid from the sample extraction vial was removed and filtered using a Millex-GV 0.45-μm filter (Millipore, Bedford, MA, USA).

2.2. Sample preparation and preconcentration from lamotrigine tablets

A pestle and mortar was washed with 10 ml of a 1-mg/ml EDTA aqueous solution and the washings collected in a scintillation vial to provide an extraction blank. Tablets equivalent to 200 mg of lamotrigine were powdered using the pestle and mortar; 10 ml of the 1 mg/ml EDTA aqueous solution were added to the mortar and the suspension carefully mixed and emptied into a scintillation vial. Both vials were placed on a mechanical shaker for 30 min; 10 ml of 1,2-dichloroethane was then added and both vials shaken for a further 30 min. Both vials were then centrifuged twice at 2500 rpm for 30 min before the 1,2-dichloroethane layers were carefully removed by disposable Pasteur pipette and transferred to clean scintillation vials. The contents of the two vials were concentrated under a stream of dry nitrogen to approximately 500 μl. The contents of the sample extraction vial were filtered using a Millex-GV 0.45-μm filter.

2.3. Normal-phase chromatography

A total of 10 μl of a 10-ng/μl solution of 14W80 in the mobile phase was injected into the normal-phase HPLC system as a standard. A 100-μl volume of mobile phase was injected after the standard to ensure there was no carry-over.

A 150-μl volume of the extraction blank, followed by 150 μl from the sample extraction (prepared from bulk powder or extracted from tablets), was injected into the normal-phase HPLC system.

Two Waters (Milford, MA, USA) 510 pumps with automated gradient controller were used together with a Waters 712 WISP autosampler and a Waters 490E programmable multiwavelength detector. The column temperature was maintained at 30°C using an oven unit obtained from Jones Chromatography (Hengoed, UK). The semipreparative Zorbax Sil column (250×9.4 mm) was purchased from DuPont (Wilmington, DE, USA). The mobile phase was 1,2-dichloroethane–methanol (90:10, v/v) at a flow rate of 2.5 ml/min; detection was by UV 340 nm with a sensitivity of 0.100 absorbance unit full scale (AUFS) range. Quantitative evaluation of the chromatograms was made using a Multichrom data acquisition and analysis system (VG Data Systems, Altrincham, UK). The fractions corresponding to 14W80 were collected manually into scintillation vials from: (1) injection of the mobile phase as a machine blank (150 μl); (2) the extraction blank (150 μl); followed by (3) the sample extraction (150 μl). The fractions collected were blown to dryness under a stream of dry nitrogen and redissolved in 200 μl of the reversed-phase chromatography mobile phase (methanol–water (55:45, v/v) + 0.1 M ammonium acetate).

2.4. RP-HPLC–mass spectrometry method with TSP ionisation

A total 2 μl of a 100-pg/μl solution of 14W80 in the mobile phase was injected into the reversed-phase HPLC system as a standard. A 100-μl volume of mobile phase was injected after the standard to ensure there was no carry-over.

The redissolved fractions were transferred to autosampler vials, and 100 μl injected into the HPLC–MS system. A Hewlett-Packard (Waldbronn, Germany) Model 1050 pump unit, ultraviolet detector and autosampler were used. The reversed-phase column was a Zorbax Rx ODS (150×4.6 mm) (DuPont). The mobile phase flow rate was 1.0 ml/min and the column temperature was 30°C. The mobile phase was methanol–water (55:45, v/v) + 0.1 M ammonium acetate. The

effluent was monitored by UV at 340 nm and also by a thermospray interface to a Thermoquest (Manchester, UK) TRIO-1000 mass spectrometer. Positive thermospray ionisation was applied for the detection of 14W80 which was detected by single ion monitoring of the protonated molecular ion $(M + H)^+$ at m/z 256. The thermospray conditions were: source temperature, 230°C; nozzle temperature, 220°C; and a repeller voltage of 140 V.

The 14W80 standard must be stored in the fridge as lamotrigine can be formed from 14W80 on standing in aqueous solution.

3. Results and discussion

3.1. Extraction from bulk powder

The route indicative impurity, 14W80, was detected at the 0.001% level in bulk lamotrigine powder using the method described in Section 2.1 When this method was used to extract 14W80 from lamotrigine tablets, no 14W80 was observed. When the extract was spiked with 1 μg of 14W80, only 2–3 ng were recovered. It was discovered that the presence of metal ions strongly affected the extraction efficiency and so the EDTA extraction method was established.

3.2. Shake flask partitioning extraction with EDTA

The purpose of the application of EDTA in the water extracting solvent was to exclude the 14W80 from forming any chelate complex with the iron or magnesium present in the tablet formulations. A higher concentration of EDTA (10 mg/ml) did not increase the extraction efficiency. The first 30 min of shaking the tablet powder with water helps to disintegrate the tablet components fully. The addition of 1,2-dichloroethane and the further 30 min shaking helps to partition the non-polar 14W80 into the organic phase. An emulsion/suspension is formed with the aqueous, organic and solid components, the separation of which requires the centrifuging procedure to be repeated twice. The solid tablet components are usually suspended between the upper aqueous and lower organic layer, and care must be taken when removing the organic layer not to include any of the solid component.

3.3. Normal-phase HPLC

Typical HPLC-UV chromatograms, obtained from normal-phase chromatography of the standard 14W80 and for the sample extract from lamotrigine tablets are shown in Fig. 2. The peak at 8.15 min in Fig. 2a corresponds to 14W80. The large peak at 17.2 min on the chromatogram of the sample extract (Fig. 2b) corresponds to lamotrigine.

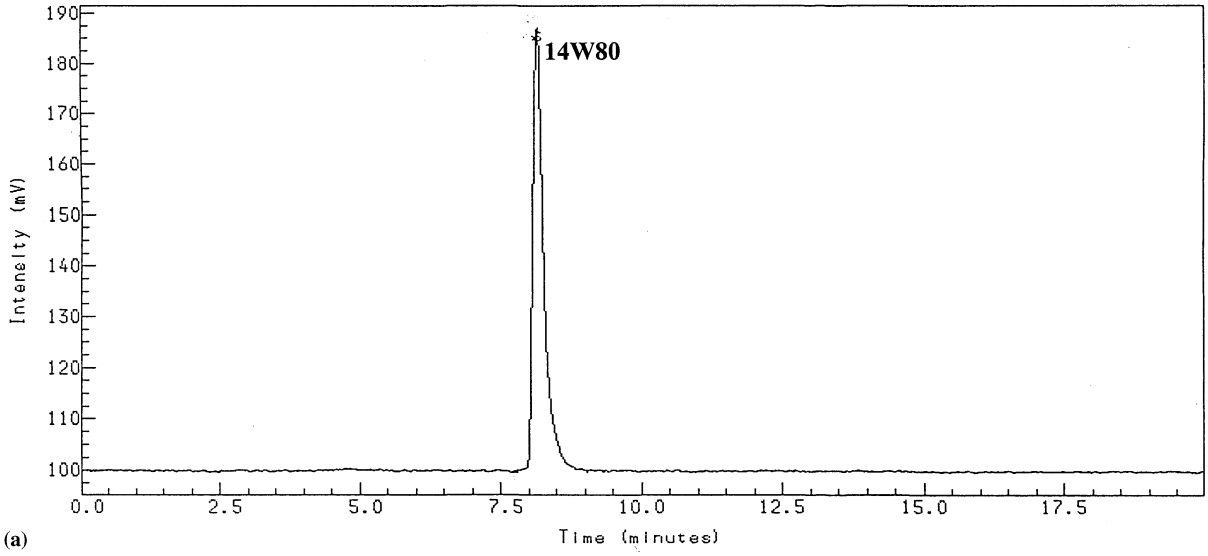
A semi-preparative scale column was used in preference to an analytical scale column due to the greater loading permissible.

3.4. Reversed-phase HPLC–TSP-MS

The fraction corresponding to the 14W80 peak in the normal-phase chromatography was collected (see Fig. 2). After evaporation of the solvent under nitrogen flow, it was re-dissolved in 200 μl mobile phase applied in reversed-phase chromatography (55% methanol and 45% ammonium acetate buffer). Then the blank, a 200-pg 14W80 standard solution, and the re-dissolved extract were injected to the RP-HPLC–TSP-MS system. The mass spectrometric detection was set up for single-ion monitoring at m/z 256. Both the lamotrigine and the 14W80 provide a protonated molecular ion $(M + H)^+$ 256, so the only differentiation between the two compounds is provided by the different retention times of lamotrigine and 14W80.

Typical RP-HPLC–TSP-MS ion chromatograms of a 200-pg standard injection of 14W80 and the redissolved fraction from the normal-phase HPLC of the sample extract from lamotrigine tablets are shown in Fig. 3.

All the structures shown in Fig. 1 have the same molecular formula and hence the same molecular weight. They appear with the single ion monitoring of m/z 256 ion-current chromatograms at different retention times. The other peaks appearing in the chromatogram of the ex-



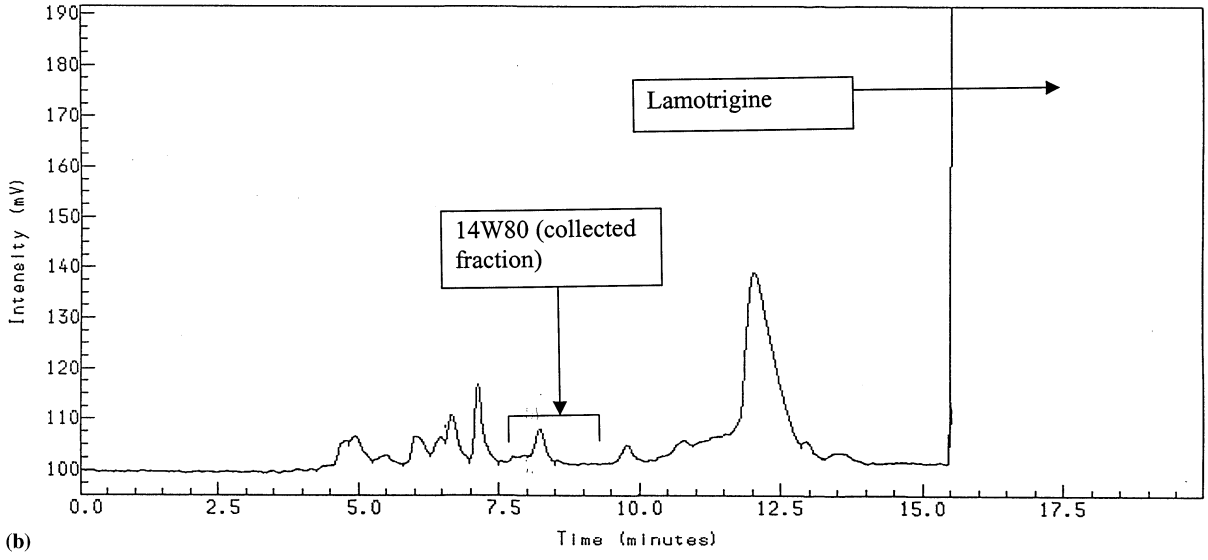


Fig. 2. Normal-phase HPLC chromatogram of 14W80 standard solution (200 pg). Column, 250 × 9.4 mm Zorbax Sil; column temperature, 30°C; mobile phase, 1,2-dichloroethane–methanol (90:10, v/v); flow rate, 2.5 ml/min; detection, UV 340 nm. (b) Normal-phase HPLC chroatogram of the extracted sample extraction from lamotrigine tablets. Column, 250 × 9.4 mm Zorbax Sil; column temperature, 30°C; mobile phase, 1,2-dichloroethane–methanol (90:10, v/v); flow rate, 2.5 ml/min; detection, UV 340 nm.

tract are due to lamotrigine and the geometrical isomer of 14W80 (13W80, **3** in Fig. 1). The peak at 2.8 min in Fig. 3a is due to lamotrigine. The peak at 3.4 min in Fig. 3b is due to 13W80 (**3** in Fig. 1); the peak at 9.5 min in Fig. 3b is due to the extracted 14W80.

3.5. Detection limit

The detection limit of 14W80 when analysed by HPLC–TSP-MS with single ion monitoring was determined by injecting progressively less 14W80 until the signal-to-noise ratio decreased to 3. The detection limit of HPLC–TSP-MS determined by this method was shown to be 50 pg. A system

suitability test of 200 pg 14W80 could reproducibly be achieved as can be seen in Fig. 3a.

3.6. Recovery study

The method presented here is not strictly quantitative. However the extraction efficiency was calculated by spiking the sample extract with varying amounts of authentic 14W80 at the earliest stage, i.e. added to the bulk powder in the extraction solvent. An extraction efficiency of 35% can be achieved from the powder but much lower extraction efficiency can be expected from tablets due to the possible and variable interaction with the tablet components.

In conclusion a very sensitive and selective method has been developed for the detection of a route indicative impurity present in lamotrigine powder or tablets, which enables the detection of 14W80 in the 50–100 ppb range.

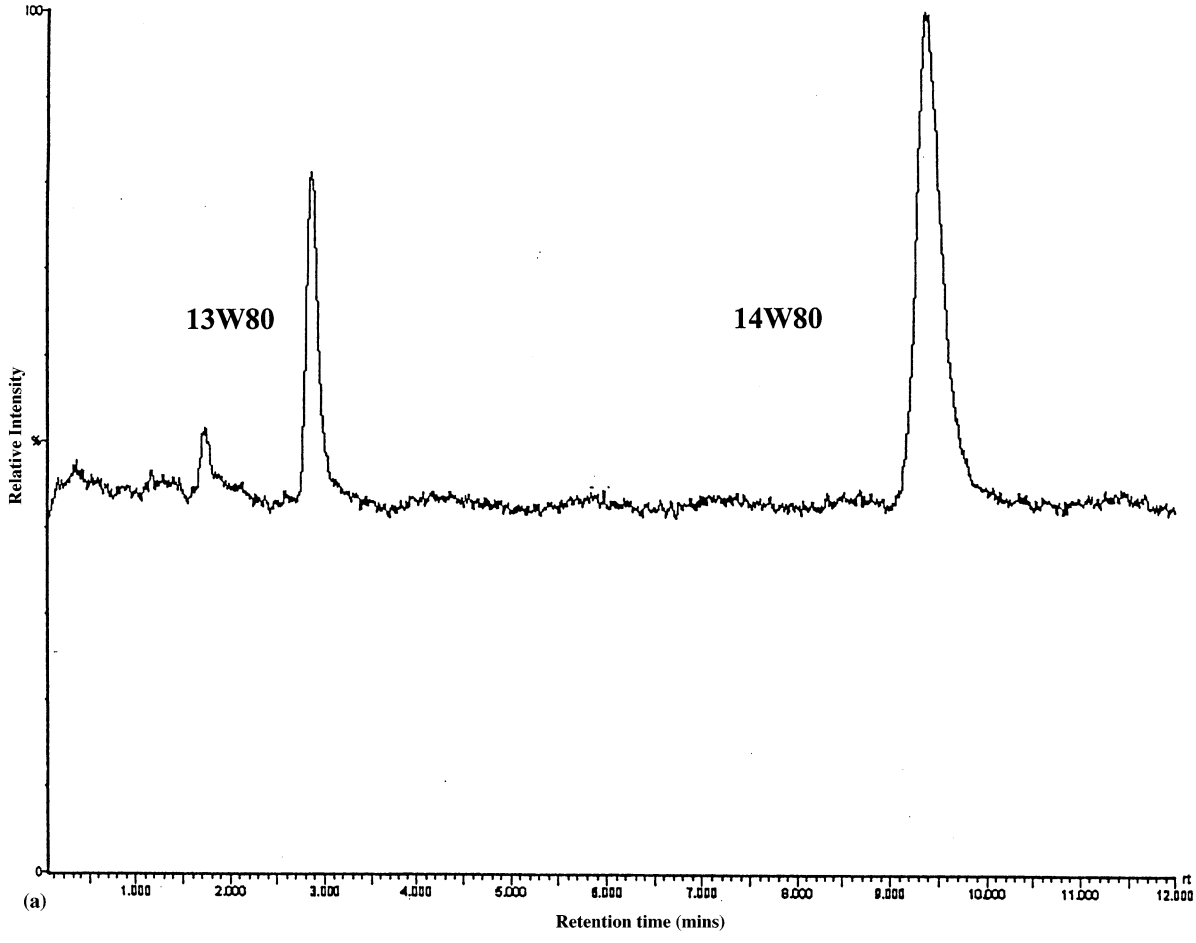


Fig. 3. RP-HPLC–TSP-MS chromatogram obtained by single ion monitoring at m/z 256 of 14W80 standard solution (200 pg). Column, 150 × 4.6 mm Zorbax Rx ODS; column temperature, 30°C; mobile phase, methanol–water (55:45, v/v) + 0.1 M ammonium acetate; flow rate, 1 ml/min. MS conditions: source temperature, 230°C; nozzle temperature, 220°C; and repeller voltage, 140 V. (b) RP-HPLC–TSP-MS chromatogram obtained by single ion monitoring at m/z 256 of the re-dissolved fraction extracted from the normal phase chromatography of the lamotrigine tablets. Column, 150 × 4.6 mm Zorbax Rx ODS; column temperature, 30°C; mobile phase, methanol–water (55:45, v/v) + 0.1 M ammonium acetate; flow rate, 1 ml/min. MS conditions: source temperature, 230°C; nozzle temperature, 220°C; repeller voltage, 140 V.

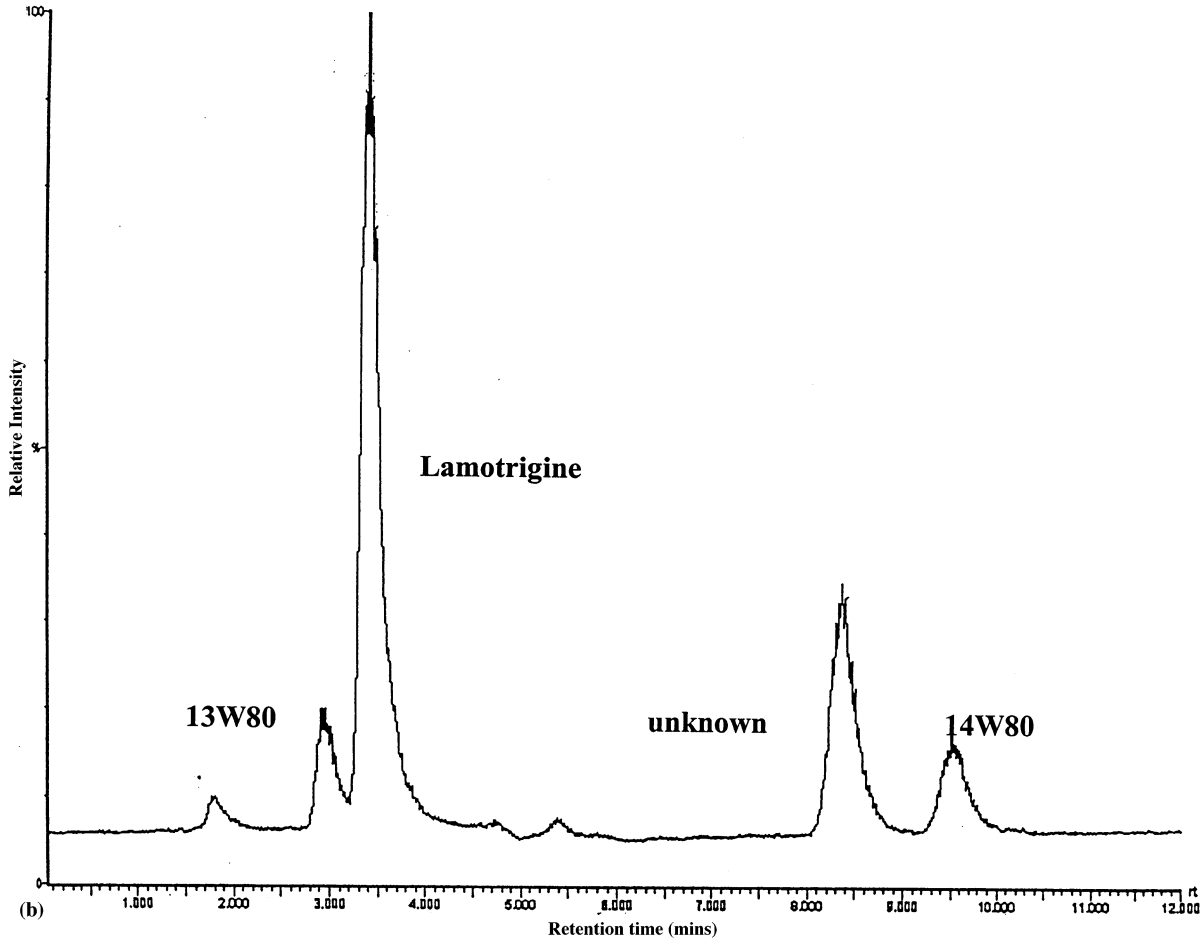


Fig. 3. (*Continued*)

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